

Interactive comment on “16S rRNA gene metabarcoding reveals a potential metabolic role for intracellular bacteria in a major marine planktonic calcifier (Foraminifera)” by Clare Bird et al.

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The authors wish to thank referee #2 for their time and contribution to this enjoyable discussion, and address her/his points below.

(1) Amplicon sequencing does not exactly show the quantity of DNA in the cell. The authors used amplicons, which were amplified by PCR, for 16S rDNA sequencing. In this process, the primers never randomly attach to DNA molecules. Even though 16S amplicon shows high abundance of DNA sequences of the cyanobacteria (Result 3.3), this does not mean high abundance of cyanobacteria in the foraminiferal cell.

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A large variation (37–87%) of the abundances of the cyanobacterial DNA sequences possibly shows a bias of PCR amplification. The authors removed some contaminated sequences from the samples based on the three negative controls (Method 2.6.1). However, we still find the sequences of the class Alphaproteobacteria in Fig. 3. What are they? Are they contaminants, preys, or symbionts?

The authors agree with referee #2 that amplicon sequencing is not a quantitative method and hence we have presented our data as proportionality bar charts and not absolute numbers. However, the next-generation sequencing methods employed in this study are currently routinely used to assess bacterial populations in a variety of environments. We accept that primer bias is a general and acknowledged weakness in any amplification procedure but it is not a weakness specific to our study per se.

As discussed with Yoshiyuki Ishitani, author of SC1 (many thanks to this contributor), the primer set employed in this study is that designed and used by the Earth Microbiome Project (Gilbert et al., 2010). The biases in this primer set are well known and have recently been corrected for (Apprill et al., 2015; Walters et al., 2016; Parada et al., 2016). The primer set has been tested with communities of known species composition (Parada et al., 2016) and compares well with FISH results (Apprill et al. 2015) giving a good representation of the bacterial assemblages targeted. The bias in this primer set does not include an over-amplification of *Synechococcus*. This is further demonstrated in the second foraminifera species presented in this study, *N. dutertrei*, where *Synechococcus* sequences were present but in a very low proportion. There is, therefore, no evidence to support the implication by referee #2 that this primer set is biased in favour of *Synechococcus*.

Instead, we suggest that the variation in the proportion of *Synechococcus* OTUs (37%–87%) in the dataset is exactly what we would expect given the sporadic nature of predation. Depending on whether a *G. bulloides* individual has recently phagocytosed bacteria or not at the point of sampling, will shift the proportions of “other” bacteria compared to the *Synechococcus* endobiont. See section 3.3 in the manuscript where

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this is stated, and also referee #2 comments in part 2 below where she/he agrees with this hypothesis.

The Alphaproteobacteria are a class of bacteria made up of a number of orders and families which encompass hundreds of species. One of the families of the Alphaproteobacteria, family Bradyrhizobiaceae is represented in our dataset by a large number of OTUs (OTUs are separated by a >3% difference in the DNA sequence). One of these OTUs was removed because it was the major contaminating OTU, as were a further 8 OTUs from the class Alphaproteobacteria (see section 2.6.1). The other OTUs within the class Alphaproteobacteria (including OTUs of the Bradyrhizobiaceae family) were not contaminants, i.e. they were not significantly amplified within the three controls and therefore they remained in the dataset and are represented in Figure 3. These Alphaproteobacteria along with all other bacteria are considered to be food because we were unable to observe any intact bacteria other than *Synechococcus* in TEM imaging.

[Changes in manuscript] In Section 2.6 we have added a fuller description of the primer set and the known biases, and discuss a lack of amplification bias towards *Synechococcus* in Section 4.1.4. We also make clear that we interpret the remaining Alphaproteobacteria OTUs as food items for the reasons laid out above via small additions to Section 4.3.

(2) The data of 16S rDNA sequences does not show “living” bacteria in the foraminiferal cell. The DNA fragments are highly retained in the cell, if the cell (foraminifer) takes bacteria through endocytosis. As mentioned in the method section 2.2, the samples were immediately put in the buffer after collection. In this case, foods were not digested in a planktonic foraminifer. If *G. bulloides* is a bacteria-feeder, the DNA fragments of foods could be detected in 16S rDNA sequencing. This hypothesis (bacteria-feeder) is also reasonable to explain why the components of 16S rDNA sequences without cyanobacteria were different among the specimens (Fig. 3).

We agree with referee #2 that 16S rRNA sequences are not conclusive of “living” bac-

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teria, and that our method of transferring samples to RNALater after collection would indeed mean that recently phagocytosed bacteria would not be completely digested. We refer back to comment 3 in our response to SC2 on this discussion forum: It has been demonstrated that DNA degradation in “dietary samples” limits the size of DNA fragments that can be successfully amplified. For investigation of prey items target fragments should be limited to ~100-250 bp. Whilst longer fragments can be utilised, they will limit the success of amplification, so that sequences will not always be obtained (see Pompanon et al., 2012 doi: 10.1111/j.1365-294X.2011.05403.x).

In our study using 16S rRNA gene metabarcoding, we have amplified a fragment of 253 bp which will therefore give us information, as referee #2 rightly points out, not only on intact bacteria but also on those bacteria that have been phagocytosed for food. Since the diet of foraminifera is not wholly known this information is also of value. TEM imaging has then enabled us to further discriminate between food and endobiont. Observations of intact and dividing *Synechococcus* cells, and of no other intact bacteria, demonstrates that the 16S rRNA genes amplified from other groups of bacteria cannot belong to an endobiont, and are most likely therefore, to be ingested prey.

We also PCR amplified partial *Synechococcus* 16S rRNA genes of 422 bp from *G. bulloides* total DNA for cloning and Sanger sequencing. This is a longer fragment than ideal for identifying prey (i.e. >250 bp) suggesting that the *Synechococcus* DNA is more intact than might be expected if it were the DNA of prey bacteria. Therefore the authors consider this to be supporting evidence (not stand alone evidence) that *Synechococcus* are living cells, endobionts, and not prey bacteria. The term “not grossly degraded by nucleases” is used in the manuscript to avoid over exaggeration of the significance of this data. [Changes in manuscript] The authors consider that additional sentences in section 4.1.1 regarding the sizes of target DNA in helping to discriminate between prey and endobiont bacteria will be a helpful addition to the discussion.

(3) The images of DAPI and TEM are not enough to support the presences of “living”

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bacteria in the foraminiferal cell. As the image of DAPI (Fig. 2) was unclear, it's difficult for me to follow the description in the result section 3.2. Although the TEM image was clear, there is no explanation about cytoplasm. Which are vacuoles? Are there phagosomes? The TEM images indicated the cell structures, which have carboxysomes and thylakoid membranes, as a character of the cyanobacteria. However, these images were not enough to certify that *Synechococcus* are living in the foraminiferal cell. As the body size of bacteria is very small rather than foraminifera, they can be remained in the foraminiferal cell. At least, the authors will need to count the number of bacteri-like cells in a planktonic foraminifer and show how these bacterial cells are universal. Moreover, I recommend the authors to use the FISH (Fluorescence in situ hybridization) method for detecting the "living" bacterial cells.

DAPI: The authors understand that the unclear nature of the DAPI images was as a result of their failure to upload properly in the first instance, an issue which has now been rectified. The DAPI images demonstrate the presence of thousands of bacteria within the host *G. bulloides* cell. The authors acknowledge that a comparison with an unstained (no DAPI) *G. bulloides* cell (suggested by referee #1) would be of benefit and will add such an image to the supplementary material. In addition, we will add an additional figure to the main manuscript of a *G. bulloides* cell observed with a TRITC filter set which excites phycoerythrin, a highly labile pigment characteristic of *Synechococcus*. It can be observed confined in bacteria-sized bright spots right across the *G. bulloides* cell. (Such an image was uploaded in reply to SC1 and can therefore be assessed by the editor and interested parties). This confirms that these multitude of bacteria are indeed *Synechococcus* and that their cell membranes are intact. Water soluble phycoerythrin would very rapidly diffuse into the surrounding aqueous milieu if the cell membrane was compromised (see section 4.1.1).

TEM: The authors acknowledge a need for more labelling on Figure 4. TEM images. We feel justified however in labelling the cyanobacteria in these images as *Synechococcus*. The genus *Synechococcus* includes all unicellular cyanobacteria of the or-

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der *Chroococcales* that lack a laminated sheath but possess thylakoids, carboxysomes and divide by binary fission in a single plane (Rippka et al., 1979) as demonstrated in the TEM image. Other names have been used for individual species but not all (e.g. *Microcystis*) have the authority of the Bacteriological Code.

The authors disagree with referee #2 that the evidence provided does not indicate living *Synechococcus*. The authors assert that since 5% of all cyanobacterial cells counted within the *G. bulloides* cell were dividing, that this is strong support for a living endobiont, particularly given that cyanobacterial cells are within the cytoplasm and not within vacuoles. We believe that the use of the term endobiont as opposed to symbiont is wholly appropriate given the evidence. We refrain from using the term symbiont (or endosymbiont), since, as referee #2 quite rightly points out, we have not yet demonstrated a benefit to either party.

FISH: As suggested by referee #2, the authors did investigate the possibility of performing FISH, as an excellent method for determining living endobionts and their metabolic activity. However, in our hands the unparalleled foraminiferal cell autofluorescence observed under fluorescence microscopy, would have drowned out the signal generated by FISH, or indeed CARD-FISH. Under such unusual circumstances, therefore, FISH was considered an unsuitable method at this time for this organism.

As referee #2 suggests, we did in fact perform cell counts, but rather than using FISH and a fluorescence micrograph, we used TEM imaging. We calculated an average *Synechococcus* cell concentration up to 4 orders of magnitude greater in *G. bulloides* compared to concentrations known in the water column in this area (section 3.4). This high count is borne out by Fluorescence microscopy, particularly using TRITC (new figure to be added).

[Changes in manuscript] Addition of supplementary image (suggested by referee #1) showing an unstained (no DAPI) *G. bulloides* cell under the DAPI filter set to be labelled supplementary figure 1 and subsequent supplementary figure numbers to be

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changed. Addition of an extra figure to be labelled Fig. 3 (subsequent figure numbers to be changed) of a *G. bulloides* cell under the TRITC filter set demonstrating the fluorescence of the pigment Phycoerythrin which is characteristic of *Synechococcus*. Additional labelling on Figure 4 TEM images (which will be relabelled Figure 5).

(4) General characters of *Synechococcus* did not prove their metabolic functions in foraminiferal cell. *Synechococcus* generally take or use nitrogen, carbon, and phosphorus. Their uptakes are different depending on species or clades. The current data and discussion (4.2) have no evidence which *Synechococcus* clade (I to IV) take these elements. Moreover, the authors should investigate intracellular distributions and assimilations of these elements. Please see one good example: Nomaki et al. (2016) published in *Frontiers in Microbiology*. Because of these reasons, the title of this MS is not supported by any results. Although this study may show the presence of bacteria in a planktonic foraminifer, it is open to question whether those bacteria are endosymbionts or not. If the authors won't show any additional information to answer the questions as mentioned above, the title, abstract, and main text should be modified to report finding bacterial cells in a planktonic foraminifer. In particular, please delete the descriptions concerning the carbon isotope of the foraminiferal shells, because no data is suggesting this topic.

The authors acknowledge that the current study does not present data on the metabolic interactions between *G. bulloides* and *Synechococcus*. Future work will elucidate the benefits to either party. Therefore we are happy to modify the title and moderate aspects of the manuscript accordingly. However, the data strongly support our conclusions that *Synechococcus* is an endobiont of *G. bulloides* Type II. As a living endobiont, the respiration of *Synechococcus* is absolutely pertinent to the carbon isotopic ratio of the *G. bulloides* shell and demands discussion. Geochemical proxies based on *G. bulloides* are particularly important for palaeoceanographic reconstructions because they provide a link between subtropical and high-latitude species. Bacterial respiration could contribute isotopically depleted respiratory carbon to the calcite shell, explaining

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part of the offset between measured and predicted $\delta^{13}\text{C}$.

[Changes in manuscript] Title changed to: 16S rRNA gene metabarcoding reveals the presence of intracellular cyanobacteria in a major marine calcifier, *G. bulloides* (planktonic foraminifera).

Minor comments:

a) Abstract (lines 3-4): Not "unusual". *G. bulloides* is one of spinose species without algal symbionts.

We used the term "unusual" to refer to two qualities of *G. bulloides*. Firstly to its lack of algal symbionts. Unusual does not imply unique, but it does mean distinct: the distinction being that many other spp. have algal symbionts. Out of the 11 spinose species living in the photic zone whose symbiont status is reported by Hemleben et al. 1989, 3 lack symbionts, but one of these (*Globigerinella calida*) has since been reported to have symbionts. Only 5 of the 11 are paleoceanographically important and out of those, *G. bulloides* is the only one without symbionts, and hence is unusual. Secondly we used the term "unusual" in reference to *G. bulloides* shell geochemistry which is universally accepted as being so, and hence we are fully justified in using the term "unusual".

b) Abstract (line 8): There is no direct and own data about the bacterial populations in the water column.

This is correct, and the authors acknowledge that having our own data for comparison would be of value. Unfortunately it was not possible to collect such data during this work. However, the SPOT site has been studied extensively over 10 annual cycles. The microbial composition and seasonal variability are well established therefore giving a clear overview of the water column assemblage (compared to a handful of snap shot samples) with which to compare the *G. bulloides* assemblage.

[Changes in manuscript] Line 8 is modified to read "To investigate the ecological inter-

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actions between *G. bulloides* and marine bacteria,”

c) Abstract (line 16): This study does not show the presence of bacterial endobiont.

We have refrained from using the term symbiont as we would agree that since no mutual benefit, or indeed a benefit to one or the other partners has been demonstrated in this study the term symbiont should not be used. However all the data presented in this study points to *Synechococcus* living inside the *G. bulloides* cell, so the term endobiont is absolutely appropriate.

d) Abstract (lines 17-19): Please delete.

The authors consider that, given that we have presented many convincing lines of argument to suggest that *Synechococcus* is an endobiont of *G. bulloides*, lines 17-19 are valid statements pertaining to the manuscript discussion.

e) Keywords: “symbiosis”, “endobiont”, and “carbon isotope” are deleted.

We accept that symbiosis should be deleted, but assert that “endobiont” and “carbon isotope” should remain, for reasons discussed above.

[Changes in manuscript] keyword “symbiosis” deleted

f) Introduction: The first two paragraphs should be omitted. Instead of them, the authors need to describe how the other studies demonstrated the presence of symbionts in Foraminifera and/or other organisms.

Referee #2 makes an excellent suggestion to describe how other studies have demonstrated the presence of symbionts/endobionts and we thank them for it. However the authors feel that the first two paragraphs on the foraminiferal contribution to the carbonate flux and their importance to palaeoceanography is of significance and therefore needs to be introduced, giving weight to the need for investigation of these organisms.

[Changes in manuscript] The first two paragraphs are revised and shortened rather than omitted. A short new paragraph is inserted, as suggested, to describe how the

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presence of protist algal symbionts has been demonstrated.

g) 2.2 Sample collection: Samples were collected from the vertical net-towing or surface water. In this case, it is very difficult to compare the hosts with bacterial components in different depths.

Off Santa Catalina Island, near the SPOT site, where the bacterial population in the water column is well documented (see point (b) above), the foraminifera were collected by scuba or net tows in the surface waters (<25m) and therefore these specimens can be compared with the well-documented bacterial composition of the water column. At Bodega Head samples were collected by vertical net tows, but these are not close to the SPOT site and therefore the two *G. bulloides* specimens collected from here and used in metabarcoding were not directly compared with the water column assemblage. However, statistical analysis (Bray-Curtis and LEfSe analysis) showed no significant difference between the bacterial composition of *G. bulloides* individuals from Catalina and those from Bodega Head.

h) 2.3 Decalcification: Have the authors decalcify and wash the cell for all specimens? Yes, see section 2.3.

i) 2.6. DNA extraction: Why did the authors use only one comparison (*N. dutertrei*)?

N. dutertrei was used as a comparison because it was the species collected at the same time and location as the Santa Catalina *G. bulloides* and therefore provides a direct comparison to both validate the methods used here and highlight that there are differences between species and also consistency across the *G. bulloides* individuals.

[Changes in manuscript] A small addition to section 3.3 clarifies why *N. dutertrei* was used as a comparison.

j) Discussion 4.1, 4.1.4: Based on amplicon sequencing, it is difficult to discuss the “abundance” of bacteria. Please see my comment (1).

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In the title of Section 4.1 we use the term “abundant” to refer to the *Synechococcus* endobiont. It is a statement not based on amplicon sequencing, but on our microscopy data and cell counts which clearly demonstrate that *Synechococcus* is indeed abundant within the *G. bulloides* cell. We refer the editor particularly to the new figure (this will be figure 3. in the revised manuscript) highlighting phycoerythrin fluorescence. In Section 4.1.4 we would like to point out that the term “abundant” is preceded by the word “relative” when referring to OTUs (which are based on amplicon sequencing) thereby indicating the proportional, and not absolute, nature of the data.

k) Discussion 4.1.3: The authors used “selective uptake” in line 28. It’s wrong, because there is no evidence.

The authors suggest that the evidence for selective uptake is the 4 orders of magnitude greater numbers of *Synechococcus* inside the *G. bulloides* cell compared to the highest numbers of *Synechococcus* found in the region. However the authors agree that it is not yet known how the *Synechococcus* arrive in the foraminiferal cell, whether the cyanobacterial population exists purely via cell division of a small number of bacteria entering the cell, or whether *Synechococcus* are phagocytosed in large numbers, and hence will modify this section accordingly.

[Changes in manuscript] The title of 4.1.3 is changed to “*Synechococcus* cells accumulate in the *G. bulloides* cytoplasm” to remove reference to *Synechococcus* being specifically taken up from the water column. The term “selectively accumulated” is replaced by “accumulate” in the first sentence and the word “selective” is removed from line 28. An additional sentence is added to stress that it is not yet known how the *Synechococcus* cells accumulate in the cytoplasm in order to move away from the concept of “selective uptake”.

l) Discussion 4.1.4: A visual coloration of planktonic foraminiferal cytoplasm does not demonstrate the characters of *Synechococcus* clades

We assume that this is in reference to Discussion section 4.1.5. The referee is correct.

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The colouration of the foraminiferal cytoplasm isn’t evidence of *Synechococcus* clades; the systematic evidence from both 16S and *rbcl* genes is: these are sequences predominantly from Clade IV *Synechococcus*.

[Changes to manuscript] An additional few words have been added to clarify that not all Clade IV *Synechococcus* have yet been characterised for pigments, which is further clarified in the following Section 4.2.

m) Figure 2: Unfortunately, Fig. 2 was somehow incomplete. Especially, I cannot find black arrows in Fig. 2a

The authors understand that this was a technical issue regarding the initial upload of the manuscript. This has since been rectified and Figure 2 is now complete.

Interactive comment on Biogeosciences Discuss., doi:10.5194/bg-2016-406, 2016.

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